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New breast cell lines and cell strains were developed from breast cancer tissues, uninvolved tissues from cancer patients and normal tissues from reductive mammoplasty patients. Two different approaches were tried to generate breast cell lines with malignant properties: (a) transfect the breast cells with a plasmid containing mutated *ras* oncogene and (b) treat a normal (non-malignant) breast cell with a mutagenic chemical *in vitro*. All the cell strains/lines developed were added to the breast cell repository. Seventeen cultures passed through the second subculture and were cryopreserved. Of seventeen, six were classified as cell lines and the remaining eleven as cell strains. Some of the established cell lines have been partially characterized with respect to karyotype, the presence of the H-*ras* and p53 genes, expression of specific gene markers, and cumulative population doubling levels. Preliminary results show significant cytotoxic effects on an established cancer cell line by two anticancer agents, taxol and 5-fluorouracil. Additional cell cultures will be developed and characterized during the grant extension period.

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N/A For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

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____ In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Thomas Peel 9/27/96

PI - Signature Date

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INTRODUCTION

This grant (DAMD-17-94-J-4395) was awarded to BRFF in August 1994 for a two year period. BRFF requested a one-year extension without additional funding on July 10, 1996 and the extension was approved on August 1, 1996. One of the requirements of this extension was the submission of this *second* Annual Report and a Final Report at the end of the third year.

Nature of the Problem

The main objectives for this grant are (i) to develop a number of new human breast cell lines (the aim is to establish 25 cell lines) from cancer patients of divergent ethnicity and different stages of the disease, and (ii) to operate a full service repository for such cells and genomic DNA in order to make them available to the research community for further breast cancer research. The nature of the work to achieve the two aspects supported by this grant is quite different. Establishment of cell lines requires considerable research and development (R&D) work while the implementation of the cell repository requires appropriate managerial and administrative effort.

Background of Previous Work

Availability of well-characterized human mammary cells to the research community will definitely be a catalyst for stimulation of such studies using modern cellular and molecular biological concepts and other emerging technologies. It has been fairly difficult to establish human breast cancer cell lines as evidenced by the paucity of the reported development of aneuploid cancer cell lines. There are a number of plausible explanations (36,39,46,57) for this difficulty, and some of the variables have been addressed in the work performed during the two year period.

During the first year, we developed 10 cell cultures from human breast cancer tissues. Six of these cell cultures were classified as cell strains because they had not been in continuous culture for more than 10 subcultures . These cell strains were cryopreserved and in the future, some of these cell strains may become cell lines in the new culture medium being developed. In addition, the necessary infrastructure for the operation of the new BRFF cell repository is in place and is now open to the research community. All managerial and administrative aspects of the BRFF repository for breast cancer cells and genomic DNA have been implemented.

During the past year, additional information on breast cell lines (1,3-5,7,12,13,24,31,32,49) and the effect of various chemicals on breast and other epithelial cells (9,10,14,15,17,20,25,34) has been published by many authors. We have used this information in our characterization of the established cell lines and in the formulation of new serum-free culture media.

Purpose of the Present Work

The primary objective of this project is to establish, maintain and operate a repository for cell cultures and genomic DNA derived from human breast tissues. The cell collection will include both cell strains and cell lines established at BRFF using different culture media and experimental manipulations. Towards this goal, breast tissue obtained in the last two years as well as breast tissue procured in the future will be used to develop cell strains/cell lines. In addition, all cell lines developed at BRFF will be partially characterized.

Methods of Approach

The cultures developed at BRFF have utilized both classical methods that favor the growth of fibroblastic and/or normal diploid epithelial cells, as well as innovative methods that selectively grow epithelial cells. These epithelial cells are grown in special culture media supplemented with low levels of serum, as well as in serum-free media. Part of the original tissue and the cell strains are cryopreserved at the earliest possible time; cell lines from the same cell strain are expanded and again cryopreserved in larger batches.

We used two other approaches during this reporting period to obtain breast cell lines with malignant properties (or transformed phenotype). The first approach was to transfect breast cells derived from normal or cancer tissue with a plasmid containing mutated *ras* oncogene. The second approach was to treat normal (non-malignant) breast cells in culture with chemical carcinogens *in vitro*. Although these methods are unorthodox ways of producing breast cancer cells, the resultant cell lines will be useful in understanding the carcinogenic process.

Each of the continuous (immortal) cell lines are in the process of being characterized with respect to morphology, karyology, gene detection, gene expression, and growth rate. Future studies will focus on immunological detection of protein expression and tumorigenic capacity in nude mice.

In addition, the breast cancer cell strains/cell lines may be used to identify new anticancer agents.

To insure error-free operation of this valuable resource for cancer research, a body of comprehensive Standard Operating Procedures (SOP) governs the technical aspects of the various tasks and also the logistical aspects of tracking samples obtained from or delivered to collaborators.

BODY

Experimental Methods Used

Cell Culture Methods. Two methods were used to set up primary cultures of the breast cancer tissue: (i) plating single-cell suspensions or (ii) attaching small pieces of the tissue as explants on specially treated growth surfaces.

For the first method, single cell suspensions were produced by progressive enzymatic dissociation of the tissue (11) or mechanical "spilling" of the cells (33). The tissue was washed free of the transport medium, minced and suspended in 10 volumes of CTC dissociation medium (contains 0.1% Collagenase (Type I Worthington Biochemicals), 0.05%Trypsin, (Type XI Sigma), and 1% Chicken serum in HEPES Buffered Saline (HBS)) in a conical flask. The minced tissue was incubated at 37°C for 30 minutes, and then isolated cells in suspension were sieved through a cell strainer (Falcon #2350) and collected by centrifugation (100 x *g* for 5 min). Dissociation of the undigested tissue was repeated two or more times and the pooled cell suspension counted and plated as the primary culture. In some cases, the cells from the tissue could be disaggregated by cutting the tissue into small fragments.

For the second method which utilizes explant cultures (26,27), the tissue was washed free of the transport medium and cut into 1-2 mm^3 fragments with crossed scalpel blades. These fragments were then transferred to culture dishes that were "scratched" with a scalpel and coated with a coating mixture (FNC) (FNC contains bovine Fibronectin, Collagen (Type 1), and bovine serum albumin in HBS). The tumor fragments were placed on the "scratched" surface approximately 1 cm apart. A small volume of growth medium was added so that the entire surface of the dish was covered without immersing the tissue fragments in the medium. These explants were incubated at 37°C in a humidified incubator with a gas phase of 5% CO_2 in air. When substantial zones of cellular outgrowth were formed, the explanted tissues were transferred to another dish.

For both primary and subsequent subcultures (SC), cells were plated in the specific culture medium (as dictated by the experiment) and incubated at 37°C in an atmosphere of CO_2 in air . The medium was changed 3 times a week and monolayers were subcultured when they attained confluency. Anchorage-dependent cells were detached from the plastic by a dissociation medium containing Polyvinylpyrrolidone, Ethylene Glycol-bis (β-Aminoethyl Ether) N, N, N', N'-Tetraacetic Acid (EGTA), and Trypsin (PET), and the tryptic activity was inhibited by addition of Fetal Bovine Serum (FBS)-supplemented HBS. The cells were centrifuged and resuspended into the desired growth medium and a known inoculum size was then plated on FNC-coated plastic. The number of cells obtained at the subculture were recorded and used to prepare the Cumulative Population Doubling Levels (CPDL).

It was of prime importance that the cell lines be derived from the particular breast sample and not cross-contaminated by another specimen. Precautions against cross-contamination included cleaning the work surfaces with alcohol and irradiation of the Biohazard Hoods with ultraviolet light before handling each cell type, working on only one cell type at a time, and using a separate bottle of medium for each cell strain.

Description of Various Media: We established three media during the previous reporting period - BM0, BM2, and BM3. These three media have been used for cell culture during this reporting period. A summary of media composition is given below:

	BRFF-BM0*	**BRFF-BM2***	**BRFF-BM3***
Growth Factors and Hormones			
β-estradiol	0	1.00E-08	1.00E-08
Prolactin	0	1 mg/ml	1mg/ml
Dihydrotestosterone	0	1.00E-10	0
EGF	5 *n*g/ml	5 *n*g/ml	5 *n*g/ml
Insulin	5 mg/ml	5 mg/ml	5 mg/ml
Phosphoethanolamine	5.00E-06	5.00E-06	5.00E-06
Hydrocortisone	2.80E-07	2.80E-07	2.80E-07
Soy Bean Trypsin Inhibitor	10 mg/ml	10 mg/ml	10 mg/ml
Bovine Pituitary Extract	100 mg/ml	100 mg/ml	100 mg/ml

**All of the above media are based on BRFF-HPC1 developed for Human Prostatic Cell Cultures*

Cryopreservation of the breast cells. The cell suspensions were centrifuged and the pellets resuspended at a density of 4-6 x 10^6 cells per ml in ice-cold L-15 medium containing 10% FBS and 2x Gentamicin (100 mg/ml). An equal volume of ice-cold L-15 medium containing 10% FBS and 15% DMSO was added slowly (28) to the cell suspension. One ml aliquots of the cell suspension were transferred to pre-cooled cryovials, frozen slowly at a controlled rate and transferred to the vapor phase of a liquid nitrogen freezer. Samples of fresh breast tissue were also frozen and maintained at -80°C for possible future studies after establishing cell lines.

Transfection Experiments for Immortalization. Transfections (11) were carried out either using the newly developed cell strains or the explant cultures with pRSV-T DNA by lipofection as follows. For each dish, 5 μg DNA in 100 μl was mixed with an equal volume of diluted lipofectin or lipofectamine reagent (1:4 dilution) in a polystyrene tube and kept at room temperature for 15 min. It was then diluted to 2 ml with the culture medium. The monolayers were rinsed twice with the culture medium and then 2 ml of the DNA mixture was added drop-wise while gently swirling the dish. After 6-8 hr of incubation at 37°C, the medium was removed and replaced with regular medium and the transfected cultures were maintained using the routine conditions described above.

Transfection Experiments for Malignant Transformation. Transfections were carried out essentially as described above. The plasmid used in these experiments contains the mutated c-Ha-ras-1 oncogene from the T24 human bladder carcinoma and was provided by Dr. Premkumar Reddy, Fels Research Institute (48). Such cultures were subcultured at regular intervals with a view to generate new cell lines.

Morphological Studies. The cell cultures were routinely checked under a Nikon phase contrast inverted microscope and photographed. Scanned images of the photographs were captured on computer disks and printed using a laser printer.

Sterility Testing for Bacterial, Fungus Contamination. This testing was performed using BRFF SOP# T-044. Possible mycoplasma contamination was checked using molecular biology methods.

Cytogenetic analysis. Semi-confluent cultures were sent to the Cell Culture Laboratory at Children's Hospital of Michigan for karyotypic analyses. Exponentially growing cultures were treated with 0.04 μg/ml of Colcemid for 1-2 hours, trypsinized and treated with 0.0375 M KCI for 9 minutes, and fixed in 3:1 methanol:glacial acetic acid mixture. The suspension was centrifuged and washed a couple of times with fixative and finally dropped on cold wet slides as previously reported (45) Slides were air dried and stained with 4% Giemsa solution and used for the determination of ploidy distribution, chromosome counts and constitutional aberrations. For trypsin Giemsa banding (GTG), karyotypes were prepared by a modified procedure of Seabright (50). The slides were aged at 60°C on a slide warmer for 16-20 hours, immersed in 0.025% trypsin for 1-2 seconds, stained with 4% Giemsa solution for 11 minutes, washed in buffer, dried and mounted in Permount. Well banded metaphases were photographed at 800x magnification using technical Pan film 2415 (Kodak) and printed on Rapidoprint FP 1-2 (Agfa-Geavert) or were karyotyped using the AKSII image analysis system. A minimum of 5 karyotypes were prepared from these prints and arranged according to standard karyotype (38).

Genomic DNA Isolation and PCR Amplification. Genomic DNA was isolated from the cell pellets of different cell lines following an established protocol which utilizes proteinase K digestion of the cells, phenol-chloroform extraction, and ethanol precipitation (54). The quantity and purity of the genomic DNA was then checked using a Pharmacia GeneQuant DNA calculator.

PCR amplification of the genomic DNA samples was performed in order to verify that the cells had been immortalized with the pRSVT plasmid. One µg of purified DNA was utilized in PCR reactions. PCR amplification was performed using published amplimer sequences (see table below) and PCR conditions (11). Identification of the amplified gene was verified by loading 20 µl of the PCR reaction onto an agarose gel and performing electrophoresis.

PCR amplification of the genomic DNA samples was performed in order to characterize cell lines with respect to the presence or absence of specific proto-oncogenes and tumor suppressor genes. One µg of the genomic DNA was used in PCR reactions. PCR amplification of codons 12 and 61 of the *H-ras* oncogene was performed using Clontech's amplimer sets and the PCR protocol followed Clontech's suggested procedure. PCR amplification of the p53 tumor suppressor gene was also performed using Clontech's amplimer sets and suggested protocol. Specifically, exon 8 of the p53 gene was amplified. Identification of the amplified genes was verified by loading 20 µl of each PCR reaction onto an agarose gel and performing electrophoresis.

PCR for	Primers Used	Reference
pRSVT plasmid which contains the SV40-T antigen gene	combination 1: GCA-TAC-TCT-GTT-ACA-AGC-TTC TCC-AAC-CTA-TGG-AAC-TGA-TG combination 2: GCA-TAC-TCT-GTT-ACA-AGC-TTC GAA-ATG-CCA-TCT-AGT-GAT-GAT-G	Driscoll,K.E., Carter, J.M., Iype, P.T., Kumari, H.L., Crosby, L.L., Aardema, M.J., Isfort, R.J., Cody, D., Chestnut, M.H., Burns, J.L., and LeBoeuf, R.A. In Vitro Cell. Dev. Biol.-Animal 31:516-527, 1995.(11)
p53 gene - exon 8	ACC-TGA-TTT-CCT-TAC-TGC-CTC-TGG-C GTC-CTG-CTT-GCT-TAC-CTC-GCT-TAG-T	Clontech Human p53 Amplimer Panel
H-ras gene - 12th codon	ATG-ACG-GAA-TAT-AAG-CTG-GT CGC-CAG-GCT-CAC-CTC-TAT-A	Clontech Human Ha-ras/12,12 Amplimer Set
H-ras gene - 61st codon	AGG-TGG-TCA-TTG-ATG-GGG-AG AGG-AAG-CCC-TCC-CCG-GTG-CG	Clontech Human Ha-ras/61 Amplimer Set

RNA Isolation and Reverse Transcriptase-PCR (RT-PCR). RNA was isolated from human breast cells using the TRIzol reagent from Gibco/BRL and the recommended Gibco/BRL protocol.

For RT-PCR reactions, rTth polymerase from Perkin-Elmer was utilized. RT-PCR was performed using published primer sequences (see table below). RT-PCR conditions were determined empirically. RT-PCR conditions for cytokeratin 8 and cytokeratin 18 were as follows: RT reaction of 1 cycle of 60°C-30 minutes, 4°C-5 minutes and PCR reaction of 94°C-5 minutes; 30 cycles of 94°C-1 minute denaturation, 52°C-1 minute annealing, 72°C-2 minutes extension; 72°C-5 minutes. RT-PCR conditions for BRCA-1, erbB2, and Epidermal Growth Factor Receptor were as follows: RT reaction of 1 cycle of 60°C-30 minutes, 4°C-5 minutes and PCR reaction of 94 C-5 minutes; 40 cycles of 94°C-2 minutes denaturation, 52 C-2 minutes annealing, 72 C-2 minutes extension; 72°C-5 minutes. Identification of the amplified gene was verified by loading 20 µl of the RT-PCR reaction onto an agarose gel and performing electrophoresis.

RT-PCR for	Primers Used	Reference
Cytokeratin 8 (amplified fragment = 277 base pairs)	CTG-GTG-GAG-GAC-TTC-AAG-AAC GAC-CTC-AGC-AAT-GAT-GCT-GTC	Traweek, S.T., Liu, J., and Battifora, H. Am. J. Pathology 142:1111-1118, 1993. (55)
Cytokeratin 18 (amplified fragment = 135 base pairs)	AGC-CAT-TAC-TTC-AAG-ATC-ATC CTC-TGT-CTC-ATA-CTT-GAC-TCT	Traweek, S.T., Liu, J., and Battifora, H. Am. J. Pathology 142:1111-1118, 1993.(55)
BRCA-1 (exon 11) (amplified fragment = 581 base pairs)	GGG-CTG-GAA-GTA-AGG-AAA-CAT-G CAG-GAT-GAA-GGC-CTG-ATG-TAG-G	Gowen, L.C., Johnson, B.L., Latour, A.M., Sulik, K.K., and Koller, B.H. Nature Genetics 12:191-194, 1996.(18)
erbB2 (amplified fragment = 217 base pairs)	CAC-CTG-TGA-GGC-TTC-GAA-GCT-GCA-G GGA-TAT-CCA-GGA-GGT-GCA-GGG-CTA-C	Lonn, U., Lonn, S., Nilsson, B., Stenkvist, B. Cancer 75:2681-2687, 1995.(40)
Epidermal Growth Factor Receptor (amplified fragment = 202 base pairs)	AAT-ATT-CTT-GCT-GGA-TGC-GTT-TCT-GTA TTT-CGA-TAC-CCA-GGA-CCA-AGC-CAC-AGC-AGG	Patel, V.G., Shum-Siu, A., Henifold, B.W., Wieman, T.J.,and Hendler, F.J Am J. Pathology 144:7-14, 1984. (44)

Preliminary In Vitro Carcinogenesis Experiments: A focus assay was used in the preliminary *in vitro* chemical carcinogenesis experiment with 7,12-dimethyl benz(a)-anthracene (DMBA; 10 ng/ml), 20-methylcholanthrene (MCA; 100 ng/ml), and N-methyl-N^1-nitro-N-nitrosoguanidine (MNNG; 1 µg/ml). BRF-97TA cells at SC 14 were seeded in the serum-free BM0 in FNC-coated dishes at 6 x 10^4 cells per 60 mm dish or the cells were seeded in BM0 supplemented with 5% FBS in non-FNC coated dishes. Twenty-four hours after plating, the cells were treated with carcinogens and maintained in culture for a maximum of 2.5 weeks. The dishes were rinsed with Phosphate Buffered Saline (PBS), then rinsed with PBS/10% methanol, fixed with 100% methanol for 15 minutes, and stained with 10% Giemsa in HBS. Dished containing "piled up" foci were photographed using an Ambis Image Analyzer. Live (unfixed) "piled up" foci from MNNG-treated dishes were also subcultured at regular intervals in order to generate additional cell lines.

Preliminary Study on the Effect of Anticancer Agents on Breast Cancer Cell Lines: A microassay in 96-well plates was employed using Sulforhodamine B (SRB) staining as the endpoint to test the effects of Paclitaxel (Taxol) and 5-fluorouracil (5-FU) on a breast cancer cell line. The SRB Assay described by Skehan *et al.* (52) was used after minor modifications to determine the SRB OD Units of cells grown under high density conditions. The plating of cells and drug treatment of the cells are similar to the published conditions. After 7 days in culture, the cells were fixed directly with cold TCA (50%) for 60 minutes, rinsed 5 times with tap water, and stained with 0.4% Sulforhodamine B (w/v) for 15 minutes. This was followed by rinsing the stained cells 5 times with 1% acetic acid.

The plates were air-dried completely and examined under an inverted microscope for an overall qualitative examination. The dye from dried cells was solubilized with 200 µl of 10 mM Tris base (pH 10.5) for 5 minutes on a gyrotory shaker. The optical density of each well was read on a Microplate Reader (MR 600; Dynatech, Chantilly, Virginia) using a wavelength of 550 nanometers. The OD readings from the MR 600 plate reader were acquired through a capture program into an ASCII file which in turn was imported into a LOTUS worksheet for analysis.

Results Obtained

During this reporting period we worked on the establishment of new breast cell cultures as well as the characterization of the breast cell lines that were established at BRFF. New breast cell cultures were developed from tissues received during the first reporting period as well as tissues received during the current reporting period.

Description of the New Human Breast Tissue Obtained During this Reporting Period:

During this reporting period, we have received a total of 20 samples mainly from Ohio State University. These included 16 cancer tissues of which 2 were lymph node metastasis. Two uninvolved tissues from cancer patients and two normal tissues from reductive mammoplasty patients were also obtained. These and other details are listed in Table 1 on the next 2 pages.

If sufficient amounts of tissue were available, a small portion was frozen for comparing the DNA profile after a cell line was established. If relatively large amounts of tissue were available, a portion was cut into small pieces and cryopreserved in a modified L15 freezing medium.

Table 1. Background of ALL Breast Tissues used for Development of Cell Cultures.

(Tissues received during the current reporting period are shown in bold)

Received On:	Tissue ID	Race	Background	Age	Tissue From:	Pathology	Cell Growth Observed
10/05/1994	BRFF029	Caucasian	Cancer Patient	33	Breast Skin	Normal	Yes
10/11/1994	BRFF030	Caucasian	Cancer Patient	69	Breast Skin	Normal	No
10/20/1994	BRFF031	Caucasian	Cancer Patient	57	Breast Skin	Normal	No
10/25/1994	BRFF032	Caucasian	Cancer Patient	34	Breast Skin	Normal	No
10/26/1994	BRFF034	Caucasian	Cancer Patient	52	Lymph Node	Metastatic	No
10/26/1994	BRFF035	Caucasian	Cancer Patient	52	Breast	Normal	Yes
10/26/1994	BRFF036	Caucasian	Cancer Patient	52	Breast Skin	Normal	Yes
12/10/1994	BRFF044	Caucasian	Cancer Patient	74	Primary Breast	Carcinoma	Yes
01/24/1995	BRFF046	Black	Cancer Patient	51	Primary Invasive	Carcinoma	Yes
01/24/1995	BRFF047	Caucasian	Cancer Patient	48	Primary Breast	Carcinoma	Yes
02/03/1995	BRFF049	Caucasian	Cancer Patient	57	Liver	Metastatic	Yes
02/06/1995	BRFF050	Caucasian	Cancer Patient	69	Ovary	Metastatic	No
02/10/1995	BRFF051	Black	Cancer Patient	72	Breast	Normal	No
02/10/1995	BRFF052	Black	Cancer Patient	72	Primary Breast	Carcinoma	No
02/10/1995	BRFF053	Black	Cancer Patient	72	Lymph Node	Metastatic	No
03/09/1995	BRFF064	Caucasian	Cancer Patient	66	Ovary	Metastatic	No
03/16/1995	BRFF065	Caucasian	Cancer Patient	32	Lymph Node	Metastatic	Yes
04/04/1995	BRFF069	Caucasian	Cancer Patient	55	Lymph Node	Metastatic	Yes
04/20/1995	BRFF071	Caucasian	Cancer Patient	44	Lymph Node	Metastatic	Yes
06/07/1995	BRFF072	Asian Indi	Cancer Patient	75	Lymph Node	Metastatic	No
06/07/1995	BRFF073	Asian Indi	Cancer Patient	75	Primary Breast	Carcinoma	No
02/15/1995	BRFF074	Asian Indi	Cancer Patient	42	Lymph Node	Metastatic	No
02/15/1995	BRFF075	Asian Indi	Cancer Patient	42	Primary Breast	Carcinoma	No
02/15/1995	BRFF076	Asian Indi	Cancer Patient	65	Primary Breast	Carcinoma	No
02/15/1995	BRFF077	Asian Indi	Cancer Patient	65	Lymph Node	Metastatic	No
07/21/1995	BRFF079	Caucasian	Cancer Patient	57	Breast Skin	Normal	Yes
07/21/1995	BRFF080	Caucasian	Cancer Patient	57	Primary Breast	Cancer	No
08/01/1995	BRFF081	Caucasian	Cancer Patient	63	Primary Breast	Cancer	No
08/11/1995	BRFF082	Black	Reduct. Mammo.	27	Breast	Normal	Yes

Table 1. Background of ALL Breast Tissues used for Development of Cell Cultures (Cont.)

(Tissues received during the current reporting period are shown in bold)

Received On:	Tissue ID	Race	Background	Age	Tissue From:	Pathology	Cell Growth Observed
08/23/1995	BRFF084	Caucasian	Cancer Patient	71	Primary Breast	Cancer	No
08/31/1995	BRFF086	Caucasian	Cancer Patient	84	Primary Breast	Cancer	Yes
08/31/1995	BRFF087	Caucasian	Cancer Patient	84	Breast	Normal	Yes
08/31/1995	BRFF088	Caucasian	Cancer Patient	40	Breast	Normal	Yes
07/11/1995	BRFF089	Caucasian	Cancer Patient	57	Lymph Node	Metastatic	Yes
09/13/1995	**BRFF096**	**Caucasian**	**Cancer Patient**	**64**	**Primary Breast**	**Cancer**	**Yes**
09/15/1995	**BRFF097**	**Caucasian**	**Reduct. Mammo.**	**39**	**Breast**	**Normal**	**Yes**
09/15/1995	**BRFF098**	**Caucasian**	**Reduct. Mammo.**	**34**	**Breast**	**Normal**	**Yes**
09/15/1995	**BRFF099**	**Caucasian**	**Cancer Patient**	**53**	**Breast**	**Normal**	**Yes**
09/15/1995	**BRFF100**	**Caucasian**	**Cancer Patient**	**45**	**Breast**	**Normal**	**No**
02/27/1996	**BRFF103**	**Black**	**Cancer Patient**	**43**	**Lymph Node**	**Metastatic**	**No**
02/27/1996	**BRFF104**	**Black**	**Cancer Patient**	**67**	**Primary Breast**	**Cancer**	**No**
03/05/1996	**BRFF105**	**Caucasian**	**Cancer Patient**	**84**	**Primary Breast**	**Cancer**	**Yes**
03/12/1996	**BRFF106**	**Black**	**Cancer Patient**	**71**	**Primary Breast**	**Cancer**	**No; Tissue Cryo-preserved**
03/12/1996	**BRFF107**	**Caucasian**	**Cancer Patient**	**51**	**Primary Breast**	**Cancer**	**Yes**
03/26/1996	**BRFF108**	**not b/w**	**Cancer Patient**	**74**	**Primary Breast**	**Cancer**	**Yes**
03/27/1996	**BRFF109**	**Black**	**Cancer Patient**	**59**	**Primary Breast**	**Cancer**	**Yes**
05/09/1996	**BRFF110**	**Caucasian**	**Cancer Patient**	**51**	**Primary Breast**	**Cancer**	**Yes**
05/17/1996	**BRFF111**	**Caucasian**	**Cancer Patient**	**71**	**Primary Breast**	**Cancer**	**No**
06/19/1996	**BRFF112**	**Caucasian**	**Cancer Patient**	**59**	**Primary Breast**	**Cancer**	**No; Tissue Cryo-preserved**
06/19/1996	**BRFF113**	**Caucasian**	**Cancer Patient**	**55**	**Primary Breast**	**Cancer**	**No; Tissue Cryo-preserved**
07/10/1996	**BRFF115**	**Caucasian**	**Cancer Patient**	**82**	**Tumor**	**Cancer**	**Yes**
07/16/1996	**BRFF116**	**Caucasian**	**Cancer Patient**	**39**	**Primary Breast**	**Cancer**	**Yes**
08/10/1996	**BRFF117**	**Caucasian**	**Cancer Patient**	**71**	**Lymph Node**	**Metastatic**	**Yes**
08/22/1996	**BRFF118**	**Caucasian**	**Cancer Patient**	**61**	**Primary Breast**	**Cancer**	**No**

Establishment of new breast cell cultures

Table 2 (on the next two pages) summarizes the breast cell cultures that were developed at BRFF during the current period as well as the breast cell lines that were characterized during this period. BRFF cell ID numbers are derived from BRFF tissue numbers (e.g., BRF109 is derived from BRFF109 tissue). When isolated cell suspensions or explant cultures were transfected with pRSVT, these transfected cell cultures are identified with a capital T after the BRF cell number. If a cell line was derived from a T24 plasmid transfection (ras oncogene), then a capital R is after the BRF cell number. Any cell line established from different experimental conditions is identified by using A,B,C, etc. Please note that SC denotes subculture or passage number.

During the current reporting period, 17 cultures had undergone a second subculture and were expanded to a sufficient number for cryopreservation. Of these 17, 6 were cultured over subculture 10 and may be classified as cell lines (BRF71TR, BRF82TB, BRF97TA, BRF97TR, BRF97TN, BRF109). The other 11 may be classified as cell strains. These cell strains will be reconstituted and grown in media to be developed during the next reporting period. During the last two years, BRFF has established a total of 10 breast cell lines and 17 breast cell strains.

Table 2. Early History of Cell Cultures Developed and/or Characterized during this Reporting Period.

Cell ID	Start Date	Transfected with Plasmid	SC1Date	SC5 Date	SC10 Date	Max # SCs	Comments
BRF69TC	04/04/1995	pRSVT	05/23/1995	07/03/1995	08/25/1995	13	Cryopreserved 15 amps
BRF71TB	04/20/1995	pRSVT	05/16/1995	06/13/1995	07/17/1995	36	Cryopreserved 25 amps
BRF71TC	04/20/1995	pRSVT	05/16/1995	06/16/1995	07/17/1995	18	Cryopreserved 23 amps
BRF71TR	04/20/1995	H-ras	05/16/1995	06/16/1995	04/04/1996	14	BRF71TC transfected with H-ras at SC#5 to create BRF71TR; cryopreserved 2 amps
BRF79TA	07/21/1995	pRSVT	08/14/1995	09/26/1995		5	Cryopreserved 1 amp
BRF79TB	07/21/1995	pRSVT	08/16/1995			4	Cryopreserved 1 amp
BRF82TB	08/11/1995	pRSVT	08/28/1995	10/05/1995	11/16/1995	11	Cryopreserved 9 amps
BRF86A	08/31/1995		09/14/1995				Discarded
BRF86TA	08/31/1995	pRSVT	09/12/1995				Discarded
BRF87	08/31/1995		09/11/1995			2	Cryopreserved 2 amps
BRF88A	08/31/1995		09/11/1995			2	Cryopreserved 3 amps
BRF88TA	08/31/1995	pRSVT	09/11/1995	11/01/1995		8	Cryopreserved 3 amps
BRF89	08/31/1995		10/02/1995	11/02/1995		6	Cryopreserved 6 amps Tissue rec'd cryopreserved
BRF96A	09/13/1995		09/25/1995				Discarded
BRF96B	09/13/1995		10/19/1995				Discard. Had beautiful epithelial cells that later died
BRF96T	09/13/1995	pRSVT	09/25/1995				Discarded
BRF96R	09/13/1995	H-ras	10/31/1995				Discarded
BRF97C	09/15/1995		10/02/1995				Discarded
BRF97TA	09/15/1995	pRSVT	10/12/1995	12/27/1995	02/15/1996	25	Cryopreserved 39 amps
BRF97TN	03/11/1996		03/29/1996	05/01/1996	06/06/1996	11	BRF97TA treated with MNNG at SC#14 to create BRF97TN; Cryopreserved 24 amps
BRF97TR	09/15/1995	H-ras	10/12/1995	12/27/1995	02/15/1996	25	BRF97TA Transfected @ SC#11 to create BRF97TR; cryopreserved 9 amps
BRF97TR	09/15/1995	H-ras	10/12/1995	02/29/1996	04/04/1996		Transfected @ SC#04; Discarded

Table 2. Early History of Cell Cultures Developed and/or Characterized during this Reporting Period (Cont.).

Cell ID	Start Date	Transfected with Plasmid	SC1 Date	SC5 Date	SC10 Date	Max # SCs	Comments
BRF98R	09/15/1995	H-ras	10/23/1995	12/14/1995		6	Cryopreserved 9 amps
BRF99	09/15/1995		10/23/1995				Transfected to get 99R
BRF99R	09/15/1995	H-ras	10/23/1995	12/26/1995		7	Cryopreserved 7 amps
BRF105	03/05/1996		07/02/1996				Discarded
BRF105TB	03/05/1996	pRSVT	06/27/1996			3	Cryopreserved 2 amps
BRF107A	07/08/1996		07/29/1996				Tissue received cryopreserved
BRF108	03/26/1996		04/10/1996				Discarded
BRF108T	03/26/1996	pRSVT	04/16/1996				Discarded
BRF109	03/27/1996		04/26/1996	05/28/1996	07/03/1996	11	Fibroblasts Cryopreserve 5 amps
BRF110A	05/09/1996		05/22/1996				Discarded
BRF110B	05/09/1996		05/28/1996	07/03/1996			Discarded
BRF110TB	05/09/1996	pRSVT	05/28/1996	07/03/1996		6	Cryopreserved 1 amp
BRF115A	07/10/1996		07/29/1996			2	
BRF115T	07/10/1996	pRSVT	08/08/1996			3	
BRF116	07/16/1996		08/05/1996			2	
BRF116T	07/16/1996	pRSVT					
BRF117	08/01/1996		08/27/96			2	Cryopreserved 1 amp

1. Description of cell cultures **derived from breast tissue**

The tissues received during this project are summarized in Table 1. Some of the tissues were cryopreserved and have not yet been reconstituted and cultured. These samples will be used in the future after we have developed better media and culture conditions.

The early history of the cell cultures developed are summarized in Table 2. Cell culture experiments were initiated in a chronological manner as the fresh tissues were received. A total of 23 primary cultures were prepared of which 3 did not go through SC1. However, one of these cultures (BRF116T) was only initiated very recently. Although cells grew well initially, in many

cases they showed terminal differentiation and could not be easily isolated from primary culture dishes.

Establishment of breast cell cultures from breast tissue resulted in three cell lines (BRF82TB, BRF97TA, and BRF109) and nine cell strains.

We established one new cancer line, BRF109, which is fibroblastic in morphology. This cell line may be useful for studying the interaction of stromal and epithelial breast cells.

We established two new normal cell lines - BRF82TB and BRF97TA. A photograph showing BRF-82TB at Subculture 10 (Fig. 1) is given in the Appendix. The BRF82TB cell line was isolated from a reductive mammoplasty tissue sample from a 27 year old Black female patient. The cells were isolated by enzymatic treatment of minced tissue. The cells were plated in an FNC-Coated T-25 vented flask and fed BRFF-BM0. After 13 days in the flask, the cells were transfected using pRSVT and Lipofectamine. The cells have been subcultured to Subculture 11 and have undergone 8 population doublings in 109 days. The BRF82TB cell line has not been used for future studies because of an extreme abnormal chromosomal profile (see below).

Photographs showing BRF-97TA at Subculture 1 (Fig. 2) and at Subculture 14 (Fig. 5a) are given in the Appendix. The BRF97TA cell line was isolated from a reductive mammoplasty tissue sample from a 39 year old Caucasian female patient. The cells were isolated as spill cells from minced tissue. The cells were plated in an FNC-Coated T-25 vented flask and fed BRFF-BM0. After 25 days in the flask, the cells were transfected using pRSVT and Lipofectamine. The cells were subcultured two days later and seeded into 2 x T-25 FNC-Coated flasks. One of the flasks showed a small colony of dividing cells. This colony was isolated and plated into an FNC-Coated T25 flask. The cells have been subcultured to Subculture 25 and have undergone 87 population doublings in 245 days.

In addition to the cell lines, we have established four cell strains from cancer tissues (BRF89, BRF105TB, BRF110TB, and BRF117) and have cryopreserved them for future use. We will reconstitute these cell strains and attempt to develop established cancer cell lines from them. Moreover, we have established five cell strains from normal tissues (BRF79TA, BRF79TB, BRF87, and BRF88, and BRF88T) and have cryopreserved them for possible future use for producing "conditioned medium" that may support the growth of fastidious breast cell cultures.

2. Description of breast cell cultures established **by transfection with H-ras oncogene**

A new strategy to establish malignant breast cell cultures was tried. One approach was to transfect cells derived from cellular outgrowth of explants of normal breast tissue with the T24 plasmid which contains the activated (mutated) H-ras oncogene. This approach yielded two cell strains, BRF98R and BRF99R. A photograph of BRF99R at SC3 is shown in Figure 3 in the Appendix.

A second approach was to transfect an immortalized normal breast cell line (BRF97TA) at SC11 with the T24 plasmid to yield the cell line, BRF97TR. After transfection with the T24 plasmid

this cell culture to date has gone through an additional 14 subcultures. Cumulative Population Doubling Levels of BRF97TA and BRF97TR are shown in Figure 4 in the Appendix. There is a slight increase in growth rate after transfection with the H-ras oncogene. Further studies will determine whether BRF97TR acquired a transformed or malignant phenotype.

A cell line developed last year from a metastatic cancer tissue after immortalization with pRSVT (BRF71TC) was retransfected at SC 5 with the T24 plasmid to develop BRF71TR. A comparative study of BRF71TC and BRF71TR may yield further insight into the possible role of the *ras* gene in breast cancer progression.

3. Description of breast cell cultures established **by treatment with mutagenic/carcinogenic chemicals.**

Another strategy to establish malignant breast cell cultures was to treat an immortalized normal breast cell line (BRF97TA) at SC 14 with a chemical carcinogen. We tested three different mutagenic/carcinogenic chemicals and the most effective chemical was MNNG. After treatment with MNNG, considerable cell death was seen in the dishes. However, foci of fast growing cells evolved from such treated cultures (Fig. 5b and 5c in the Appendix). Therefore, we were able to establish the BRF97TN cell line by using this strategy. After treatment with MNNG, this cell line has been subcultured to SC 11 and has undergone 45 population doublings in 94 days.

Some of the control as well as the MNNG-treated cells were fixed and stained to show the dramatic effects of MNNG treatment (Fig. 6 in the Appendix). There were many more "piled up" foci in dishes grown in medium that did not contain any FBS . The formation of piled up colonies is generally regarded as the manifestation of a transformed phenotype. Further studies will determine whether BRF97TN exhibits a malignant phenotype.

Characterization of Established Breast Cell Lines

During the current reporting period, we performed four different types of characterization on the established cell lines: chromosomal studies, DNA analysis, RNA analysis, and Cumulative Population Doubling studies. In addition, a preliminary experiment to test the effects of anticancer agents on an established breast cancer cell line was performed.

1. Chromosomal Studies to define the Chromosomal Profile of Cell Lines

Chromosomal studies of two cell lines (BRF82 TB and BRF97TA) were performed.

BRF82 TB (SC9). This cell line is aneuploid human female (XO/XX/XXX/XXXX/XXXXX), with most chromosome counts in the triploid range. The distribution of normal chromosomes varies widely, and there are numerous abnormal chromosomes many of which contain portions of missing normal chromosomes. No true marker chromosomes are present. Cells other than those of cell line BRF82TB SC9 are not detected in the culture.

BRF97 TA (SC6). This cell line is aneuploid human female (XO/XX), with most chromosome counts in the diploid range. There were 1 or 2 copies of N8, N9, N10, N11, N13, N14, N16, N17, N19, and N21, and 2 copies of N1, N2, N3, N4, N5, N6, N7, N12, N15, N18, N20, and N22. There were no markers present. Cells other than those of cell line BRF97 TA (SC6) are not detected in the culture. A photograph of one karyotype is given in Figure 7 in the Appendix.

2. DNA analysis to generate samples for the DNA bank and for the determination of the retention of the immortalization gene (pRSVT), protooncogene*(ras)* and tumor suppressor gene (p53)

Genomic DNA has been isolated from five different cell lines (BRF69TC, BRF71TB, BRF71TC, BRF97TA, BRF97TN) and one cell strain (BRF99R). This DNA is now stored in the DNA bank. The genomic DNA samples from BRF69TC, BRF71TB, BRF71TC, and BRF97TA were subjected to PCR amplification in order to determine if the SV40 T-antigen gene was still present. All four samples did indeed produce a PCR amplified band for the SV40 T-antigen (see Table below).

Genomic DNA samples were also used to elucidate the presence or absence of a proto-oncogene (PO), *H-ras* gene, and a tumor suppressor gene (SG), p53 gene. We observed the presence of specific *H-ras* PCR products and a specific p53 PCR product in genomic DNA samples from BRF69TC, BRF71TB, BRF71TC, BRF97TA, and BRF99R (see table below). We proposed to classify cell lines into four categories: PO+SG+, PO-SG+, PO+SG-,PO-SG- where + indicates the presence of the gene and - indicates the absence of the gene. BRF69TC, BRF71TB, and BRF71TC, BRF97TA, and BRF99R are in the PO+SG+ category.

Summary of PCR Results from Breast Cell Cultures

	BRF-69TC	BRF-71TB	BRF71-TC	BRF-97TA	BRF-99R
SV40 T-antigen Gene (pRSVT)	+	+	+	+	N/A
H-ras Gene	+	+	+	+	+
p53 Gene	+	+	+	+	+

Note: + indicates that the PCR amplified fragment was present.

3. RNA analysis to detect the expression of specific gene markers

RNA has been isolated from four different cell lines (BRF69TC, BRF71TB, BRF71TC, BRF97TA) and one cell strain (BRF99R). The RNA samples were used in Reverse Transcriptase PCR (RT-PCR) reactions to determine if specific genes were expressed. These experiments are qualitative and quantitative comparisons between cell lines cannot be made. Results from five gene markers are summarized in the table below. Cytokeratin 8 and cytokeratin 18 can be used to identify epithelial cells (30,31,37). BRCA-1 and erb-B2 have been suggested as markers for breast cancer (2,4,6,8,12,16,18,19,40,42,51). Epidermal Growth Factor Receptor (EGFR) was also tested.

Summary of RT-PCR Results for Breast Cell Cultures

	BRF-69TC	BRF-71TB	BRF-71TC	BRF-97TA	BRF-99R
Cytokeratin 8	+	+	+	+	+
Cytokeratin 18	+	+	+	+	+
BRCA-1	+	+	+	+*(3/4)	+
erb-B2	+	+	+	+	+
EGFR	+	+	+	+*(3/4)	+

*RT-PCR experiments were repeated 3-5 times. A + sign indicates that all experiments did produce amplified DNA bands. For BRF-97TA, BRCA-1 and EGFR showed amplified DNA bands in 3 out of 4 experiments.

We did try RT-PCR for the expression of estrogen receptor and for the expression of the milk protein, α-casein, but we were unable to observe any amplified products. We worked on these RT-PCR experiments for several months but since we were unsuccessful we decided to discontinue these experiments. The apparent lack of expression may be due to technical difficulties or may be due to a changed hormonal environment. This characterization is beyond the scope of this grant and should be taken up by other investigators.

4. Cumulative Population Doubling Levels

To date, the number of population doublings for the different breast cell cultures are shown below.

Cumulative Population Doublings for Cell Lines and Cell Strains Developed during the Current Reporting Period

Cell Line	Maximum Subculture Number	Total Number of Days in Culture	Cumulative Population Doublings of the Culture
BRF71TR	14	109	37.32
BRF79TA	5	67	3.19
BRF79TB	4	74	4.81
BRF82TB	11	109	7.77
BRF87	2	25	2.61
BRF88	2	22	3.58
BRF88T	8	81	10.35
BRF89	6	71	10.24
BRF97TA	25	245	87.14
BRF97TN	11	94	45.54
BRF97TR	25	243	91.15
BRF98R	6	103	11.95
BRF99R	7	143	14.44
BRF105TB	3	29	3.32
BRF109	11	103	14.66
BRF110TB	6	70	2.12

5. Testing of Taxol and 5-fluorouracil

In order to test if the established breast cancer cell lines would be useful in the identification and testing of new anticancer agents, we performed a preliminary experiment with BRF-71TC using two established anticancer agents, taxol and 5-fluorouracil (5-FU). Both these agents are used in the treatment of human breast cancer (21). The effects of taxol and 5-FU on BRF-71TC are shown in Figure 8 in the Appendix. Both the agents showed significant cytotoxic effects on BRF-71TC. However, to obtain 50% killing of BRF-71TC, approximately $1x\ 10^{-13}$ M of taxol was sufficient while $1x\ 10^{-5}$ M of 5-FU was needed. The lower concentration of taxol indicates that it is a more effective agent than 5-FU. This preliminary experiment shows that the established cancer cell lines will be useful in screening anti-breast cancer agents.

Discussion in Relation to the Goals of Research

Our goal was to produce 25 new cell lines from human breast cancer. To date, we have developed 10 cell lines and 17 cell strains. We will continue to use the reported methods in an attempt to develop additional cell lines. We plan to develop new cell culture media in the hope that the many cell strains already cryopreserved will grow into established cell lines. We also proposed to characterize any established breast cell lines. We have begun this characterization via karyotyping, PCR, and RT-PCR. We will continue this characterization in the next year.

Some of research findings have been presented as a poster at the American Association for Cancer Research (AACR) meeting in Washington, DC in April, 1996. An abstract of this poster is published in the Proceedings of AACR (29).

CONCLUSIONS

Summary of the implication of the completed research

The R&D effort for the establishment of new cell strains and cell lines progressed at the rate envisioned in the grant application. During the second year, we have developed 11 cell strains and 6 cell lines. Two of these cell lines have been partially characterized. Moreover, new cell cultures are being developed and experiments for the characterization of some of these cell cultures are in progress.

During the last two years, we have developed 10 cell lines and details on these cell lines are summarized in the table on the next page.

Breast Cell Lines Established at BRFF

Cell Line	Origin/Description	Karyo-typing	PCR of p53 and H-ras genes	RT-PCR of gene markers	Cumulative Population Doubling	Tumorigen-icity in Nude Mice
BRF-29	Normal tissue from Cancer Patient/Fibroblastic	ND	ND	ND	+	ND
BRF-69TC	Metastatic to Lymph Node/Epithelial	+	+	+	+	ND
BRF-71TB	Metastatic to Lymph Node/Epithelial	ND	+	+	+	ND
BRF-71TC	Metastatic to Lymph Node/Epithelial	+	+	+	+	ND
BRF-71TR	BRF-71TC transfected with H-ras oncogene; Epithelial	ND	ND	ND	+	ND
BRF-82TB	Normal tissue from reductive mammoplasty patient; Epithelial	+	ND	ND	+	ND
BRF-97TA	Normal tissue from reductive mammoplasty patient; Epithelial	+	+	+	+	ND
BRF-97TR	BRF-97TA transfected with H-ras oncogene; Epithelial	ND	ND	ND	+	ND
BRF-97TN	BRF-97TA treated with MNNG; Epithelial	ND	ND	ND	+	ND
BRF-109	Cancer tissue from Cancer Patient/Fibroblastic	ND	ND	ND	+	ND

A + sign indicates that these studies have been performed.

Future Work and Recommended Changes

We plan to develop new cell culture media for establishing breast cell cultures. For the development of any new serum-free culture medium for a specific cell type, it is essential to identify a basal medium that is known to support the growth of epithelial cells and then supplement it with various growth factors and hormones. During the first year of this project, we used the then available basal media such as PFMR-4 and MCDB-170 (as well as minor modifications of each) and supplemented them with various growth factors and hormones. Although the MCDB-170 based serum-free medium(SFM) has been used extensively for growing normal cells by other investigators (22,23,53) this medium was not as effective as HPC-1 (the SFM derived from modified PFMR-4 medium which was used previously in our laboratory for growing human prostatic cells) (26,27).

Although consistent cell growth was obtained from breast explants in HPC-1, no growth was seen in some breast cancer tissue explants even if the HPC-1 was supplemented with prolactin, B-estradiol, and dihydrotestosterone. Therefore, we plan to make two new basal media by manipulating the concentration of each component from both HPC-1 basal and MCDB-170 basal. For the first new basal medium (PT1 basal), the higher concentration of each component present in HPC-1 basal or MCDB-170 basal will be used. For the second basal medium (PT2 basal), the average concentration from the two basal media will be selected. We plan to check PT1 and PT2 supplemented with each of the hormones and growth factors which have been used previously. We will also examine the effect of L-Glutamine (reported to inhibit growth of breast cancer cells) (33) and various biochemicals and trace elements (34,35,41,43,47,56,58) on the growth of the breast cancer cell cultures.

In addition, the tumorigenicity studies for some of the established cell lines will be performed in the next few months.

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APPENDIX

LEGENDS

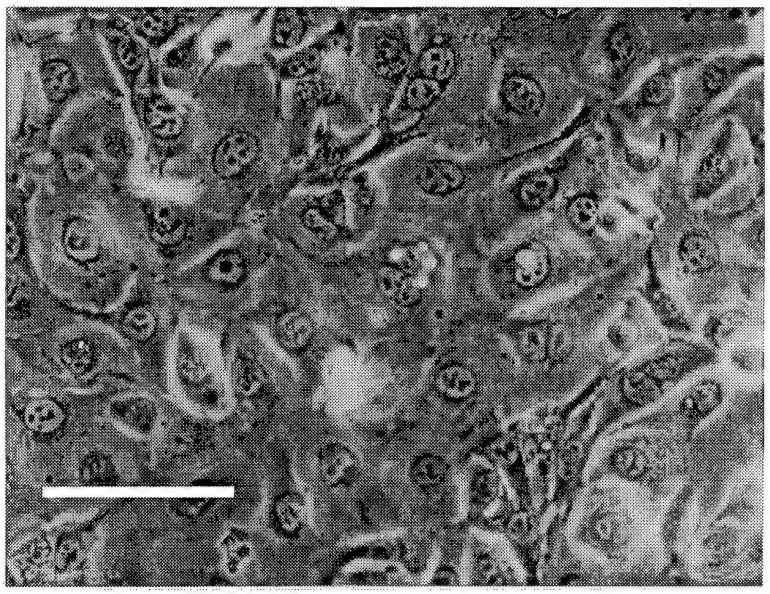


Fig. 1. Phase Contrast Picture of a monolayer culture of BRF82TB at SC 10 grown in BM0 medium.

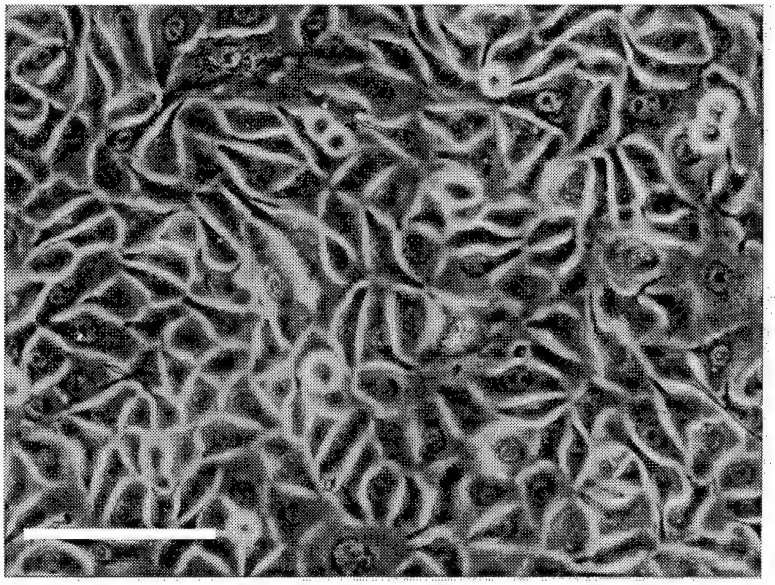


Fig. 2. Phase Contrast Picture of a monolayer culture of BRF97TA at SC 1 grown in BM0 medium.

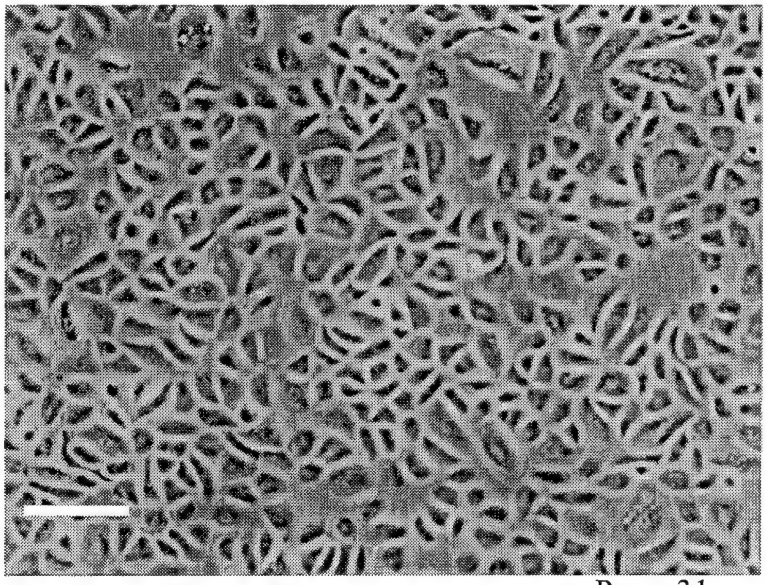
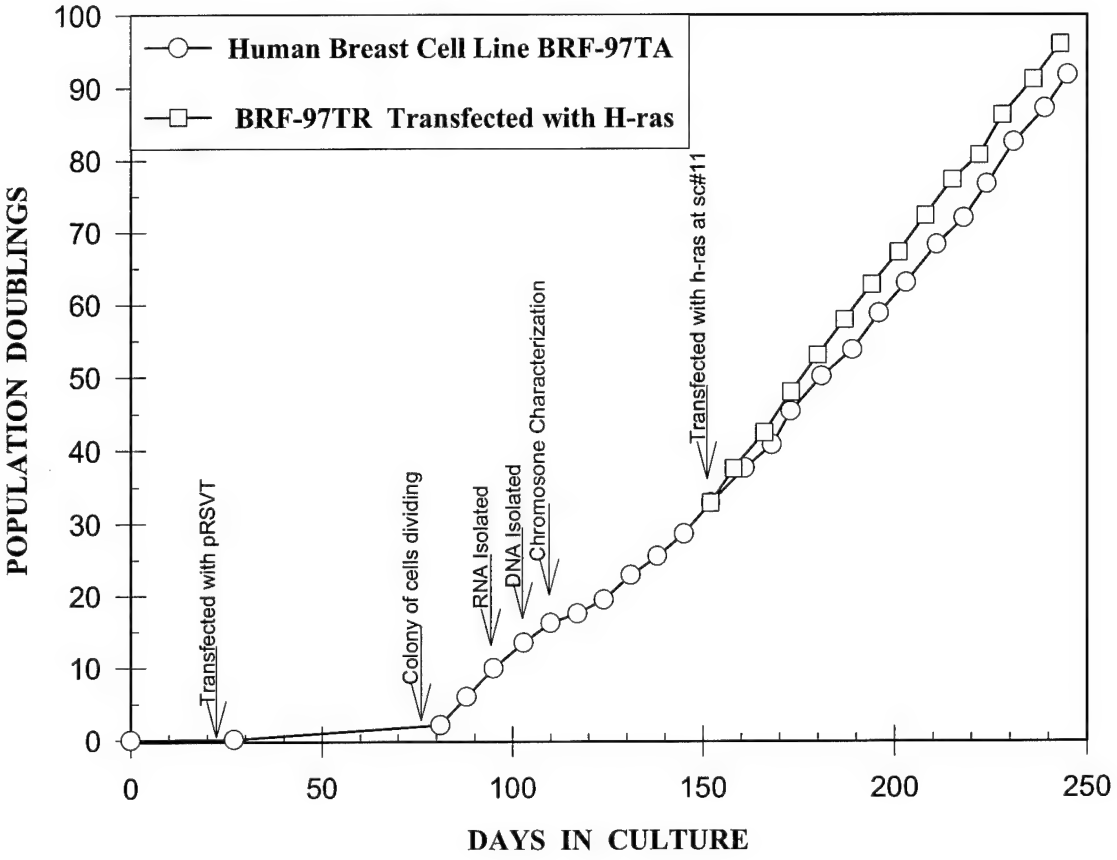


Fig. 3. Phase Contrast Picture of a monolayer culture of BRF99R at SC 3 grown in BM3 medium.

The Scale Bar represents 0.1 mm.

Fig. 4. Cumulative Population Doubling Levels BRF97TA and BRF97TR Cell Lines



LEGENDS

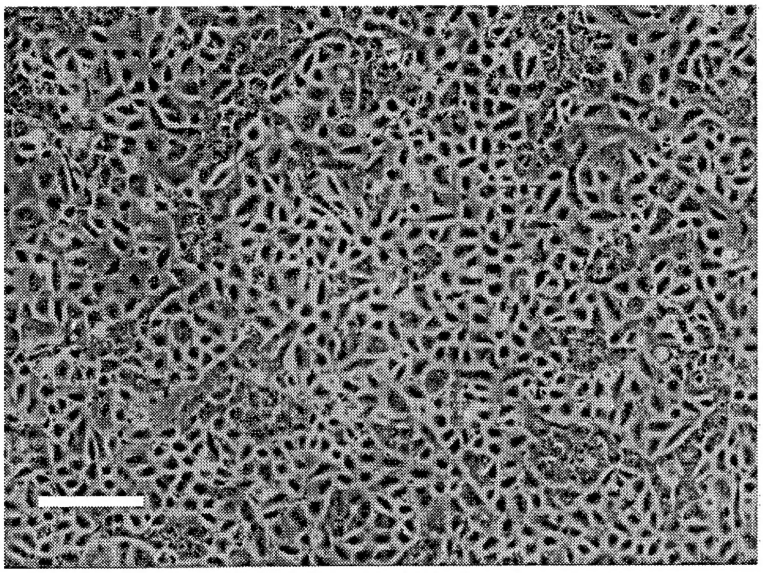


Fig. 5a. Phase Contrast Picture of a monolayer culture of BRF97TA at SC 14 grown in BM0 medium. supplemented with 5% FBS. This is a control for the BRF97TN below.

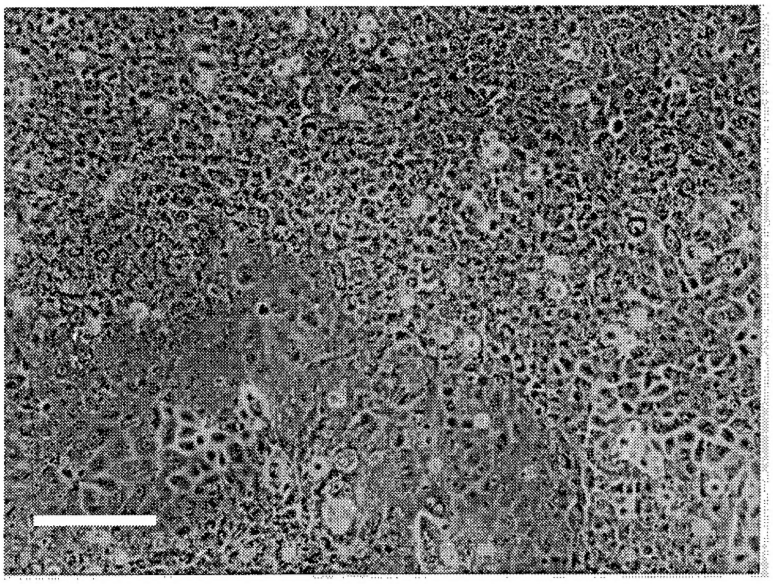


Fig. 5b. Phase Contrast Picture of a monolayer culture of BRF97TN at SC 0. This culture was derived from the BRF97TA shown above after treatment with MNNG for 10 days as described in the text.

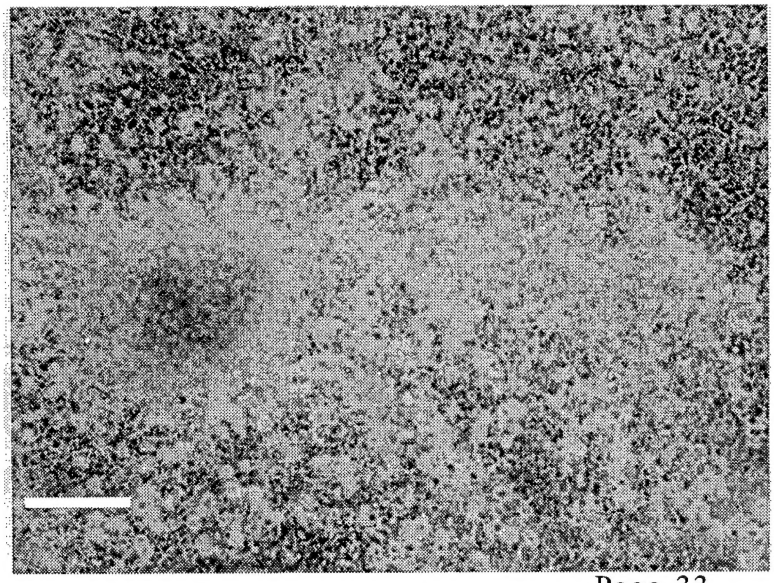
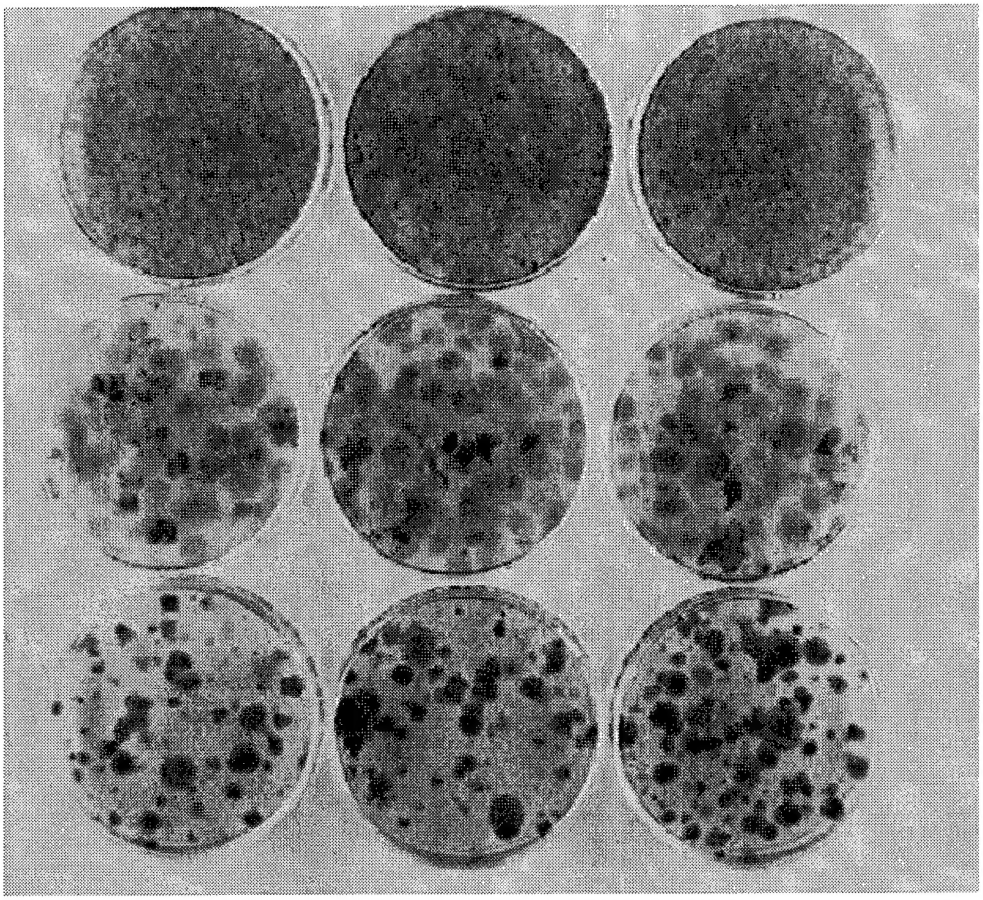


Fig. 5c. Phase Contrast Picture of a monolayer culture of BRF97TN at SC 0. This culture was derived from the BRF97TA shown above after treatment with MNNG for 18 days as described in the text.

The Scale Bar represents 0.1 mm.

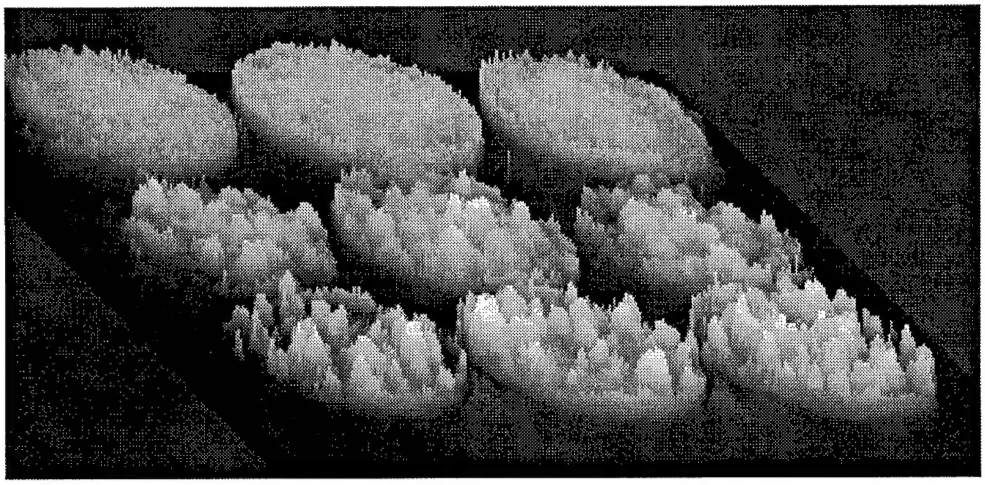
Fig. 6. Morphologically Transformed Foci of BRF-97TA Cells Treated with MNNG



Untreated cells grown and maintained in BRFF- BM0 + 5% FBS for 2 weeks;Giemsa stained.

MNNG - treated cells grown and maintained in BRFF-BM0 + 5% FBS for 2 weeks; Giemsa stained.

MNNG - treated cells grown and maintained in BRFF-BM0 on FNC-coated dishes for 2 Weeks; Giemsa stained.



Above foci as 3-D images photographed with an AMBIS Image Analyzer

Fig. 7. One of the Giemsa banded Karyotypes from BRF-97TA at Subculture 6.

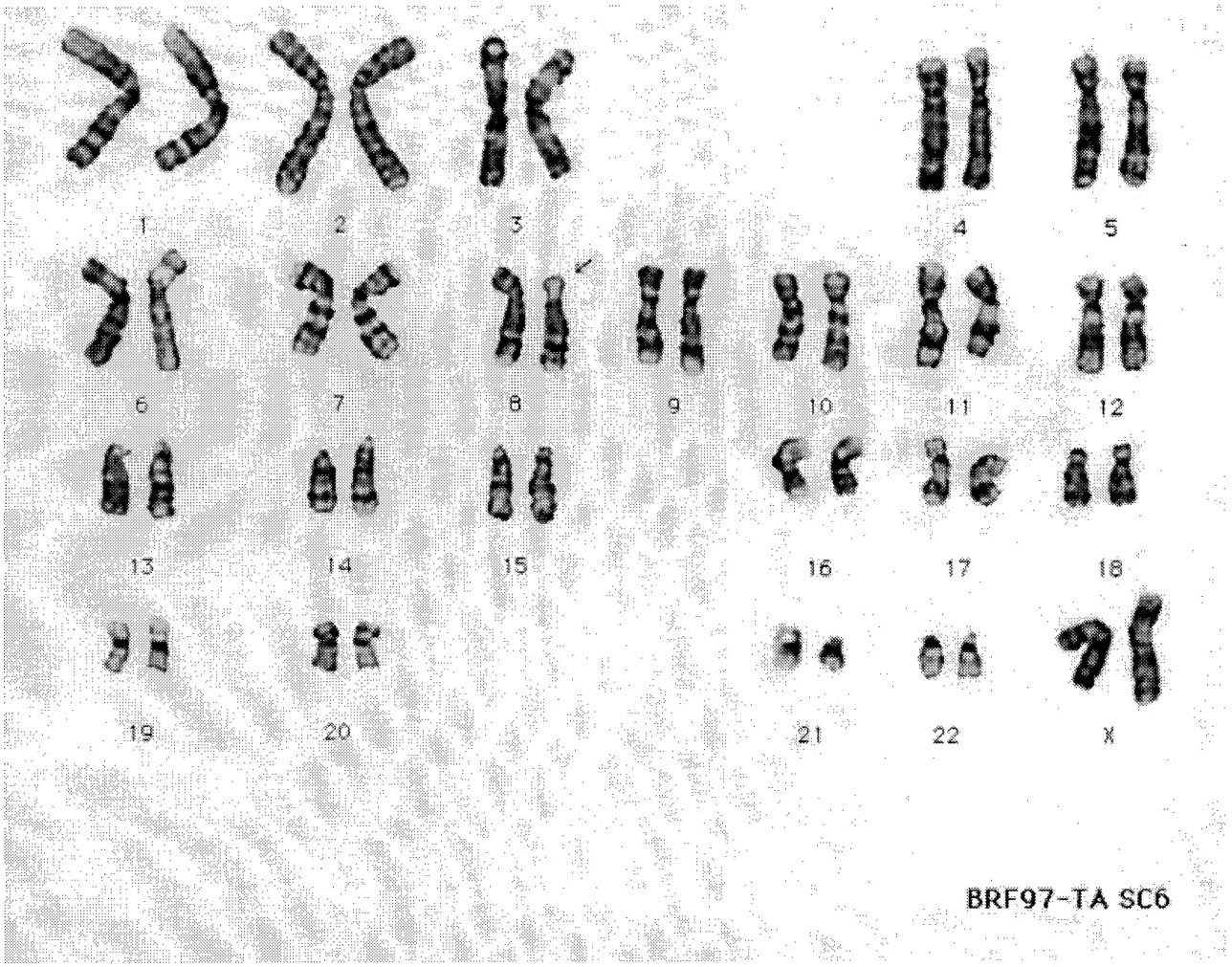


Fig. 8. Effect of Taxol and 5-FU on the growth of BRF-71TC breast cancer cell line.